Utilization of Waste Activated Bleaching Earth Containing Palm Oil in Riboflavin Production by *Ashbya gossypii*

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ABSTRACT: Waste activated bleaching earth (ABE) discharged by an oil refinery plant, which contained 40% palm oil, was used for riboflavin production by a culture of the filamentous fungus *Ashbya gossypii*. When 188 g/L waste ABE containing 75 g/L palm oil was added to the culture, 80% of the palm oil was consumed after 4 d, and the riboflavin concentration reached 2.1 g/L after 10 d of culture. This concentration was almost 1.5 times higher than for cultures grown on pure palm oil, in which case only 68% of the palm oil was consumed. Before the culture was initiated, the surface of the waste ABE was smooth and resembled clay that was covered with palm oil. After the culture, the oil content decreased to 8%. The black color of waste ABE gradually faded and turned yellow, with the ABE finally forming a yellow powder. Eighty percent of the riboflavin produced during the culture period was transferred to oil-depleted waste ABE and the waste-oil-depleted ABE had 14 mg of riboflavin/g of oil-depleted waste ABE. The waste ABE containing waste palm oil was suitable for use as a raw material for the production of value-added riboflavin. Thus, this research might provide a good model for the reuse of waste resources containing vegetable oil.

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KEY WORDS: Activated bleaching earth, *Ashbya gossypii,* oil recycle, palm oil, riboflavin, waste vegetable oil.

The manufacture of vegetable oil is a large-scale process in food and chemical industries. The worldwide consumption of palm oil in 2000 was 22.8 million tons, second to that of soybean oil. The manufacture of vegetable oil yields two kinds of waste disposal challenges: black and highly polluted wastewater, designated as oil-mill wastewater, and solid waste containing waste vegetable oil. The former is characterized by a high pollution potential, due to its high content of organic substances, and by high toxicity toward several biological systems (1–3). For the purpose of detoxifying or decolorizing this oil-mill effluent, anaerobic treatment (4), methane production (5), culturing of white-rot fungi (6) or yeast (7), and composting (8) have been used.

Among solid wastes containing waste vegetable oil, an enormous amount of activated bleaching earth (ABE) is discarded by crude oil refining industries. ABE is a very fine powder (94.5% passes through a 90-μm sieve) and its main component is silicone dioxide (79.8%). The ABE backbone is prepared by treating montmorillonite clay (represented by $Al_2O_3 \cdot 4SiO_2 \cdot nH_2O$) with mineral acids (mainly sulfuric acid) and by eluting basic components such as aluminum, iron, and magnesium. The crystal lattice of ABE is a three-layered structure of silica-alumina-silica, which confers a large specific surface area (290 m^2/g) and a large adsorption capacity because of its activated porous structure. ABE has been used to adsorb the dark color matter and odor-causing substances of crude oil, which are caused by chromophoric chloroplast-related materials with different degrees of polymerization. During this oil refinery process, ABE adsorbs 40% of vegetable oil on a weight basis and is destined to be discarded as a waste material. Japan alone annually discharges more than 80,000 metric tons of ABE. Recently, waste ABE discharged from the oil refinery process has been passed to the cement industry for incineration, but a cement manufacturer has substantial difficulty in treating waste ABE, because it contains much oil, to meet the requirements of good cement quality. Moreover, in the near future, incineration or landfill disposal will probably become impossible because of environmental restrictions, environmental pollution due to spoiling of released waste oil, lack of suitable new sites, and greenhouse effects.

There have been no reports regarding the biological treatment of solid waste containing waste palm oil. In the present study, we attempted to convert palm oil contained in waste ABE into useful value-added bioproducts using the oil-utilizing microorganism, *Ashbya gossypii*, a popular high-riboflavin producer. At present, there is a potential for production of riboflavin of more than 3,000 tons per year, with 2,500 tons of this produced by microbial fermentation because it is economically less expensive and has reduced waste and energy requirements compared with other methods.

The most popular carbon source for the production of riboflavin is vegetable oil containing high amounts of unsaturated FA, e.g., soybean oil or rapeseed oil. There are no reports on the production of riboflavin using palm oil as the carbon source, because it is difficult for the organisms that produce riboflavin to take up the large amounts of saturated FA in palm oil. However, if palm oil is identified as a suitable substrate for riboflavin production by *A. gossypii*, waste palm oil from palm oil-refining industries may be a potential substrate for riboflavin production. This study focused on the possibility of producing riboflavin through the cultivation of *A. gossypii* by using palm oil and palm oil contained in waste ABE from oil-refining industries.

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MATERIALS AND METHODS

Strain and media. Ashbya gossypii ATCC 10895 was used throughout this study. The cells were grown at pH 6 and 30°C on solid medium containing (per liter) 10 g of yeast extract (Oriental Yeast Co., Tokyo, Japan), 10 g of glucose, 3 g of glycine (Kanto Kagaku, Tokyo, Japan), and 20 g of agar. After 2 d, the plates were stored at 4°C. A subculture was carried out every 4 wk. Inocula for flask cultures were grown in a modified seed medium (9) consisting of (per liter) 30 g of corn steep liquor (CSL) (Sigma Chemical Co., St. Louis, MO), 9 g of yeast extract, and 15 g of soybean oil. The medium was adjusted by addition of 1 M KOH to pH 6.8 and dispensed into 500-mL Erlenmeyer flasks in 100-mL portions. The seed culture was carried out at 28°C on a rotary shaker (TB-25R; Bio Shaker, Takasaki Scientific Instrument Corp.) at 200 rpm for 40 h.

Riboflavin production by A. gossypii *using waste ABE containing palm oil.* For riboflavin production in flask cultures, 2.5 mL of seed culture was inoculated into 500-mL Erlenmeyer flasks containing 50 mL of production medium (9) consisting of (per liter) 30 g of gelatin (Wako Pure Chemical Industries, Osaka, Japan), 60 g of CSL, 1.5 g of glycine, 1.5 g of KH_2PO_4, 4.4 mg of $CoCl_2$, 17.9 mg of $MnCl_2{\cdot}4H_2O$, 44.2 mg of $ZnSO_4{\cdot}7H_2O$, and 10.3 mg of $MgSO_4{\cdot}7H_2O$. The concentrations of gelatin, CSL, and glycine had been optimized beforehand, making them suitable for riboflavin production from waste ABE containing palm oil. These ingredients were dissolved in 1000 mL of distilled water. The pH was adjusted to 6.8, and 50 mL of medium was then dispensed into 500-mL Erlenmeyer flasks. To each flask, various amounts of palm oil were added as the carbon source. The palm-oil-containing waste ABE (Mizusawa Industrial Chemicals Ltd., Niigata, Japan) was used as a resource for the carbon source and was stored at 4°C to avoid rancidity of the vegetable oil. In the case of the culture using waste ABE as the carbon source, because its oil content by weight was 40%, the amount of waste ABE to be added to the culture was calculated by using the following equation:

$$\text{waste ABE concentration (g/L)} = \frac{\text{palm oil concentration (g/L)}}{0.40} \quad [1]$$

The culture was performed at 28°C on a rotary shaker at 200 rpm for 7 d.

Analytical methods. Before sampling, the culture broth was mixed well. A 5-mL sample was drawn and kept at –20°C in a freezer for riboflavin assay. The residual palm oil concentration was measured by the solvent-extraction method (10). In the case of palm-oil-containing waste ABE, a 10-mL sample was taken in the homogeneous phase. The sample was added to 10 mL of *n*-hexane in a screw-capped 50-mL Falcon tube, and the tube was shaken vigorously for 2 min and then centrifuged at 500 × *g* for 15 min. The upper layer was removed and dried by using a vacuum evaporator and then placed in an oven at 80°C for 3 h to determine the weight of the extracted palm oil.

For quantitative determinations of FA, the sample was dissolved in 1 mL of chloroform, and a 4-µL aliquot was injected into a glass column (3 mm × 2 m) packed with 5% Advans DS on 80/100-mesh Chromosorb W (Shimadzu Corp., Kyoto, Japan) in a gas chromatograph (GC-14B; Shimadzu) equipped with an FID. The detector and injection port temperatures were 250 and 240°C, respectively, and the column temperature was maintained at 190°C. Quantitative determinations were performed using FAME as standards. Methyl pentadecanoate was used as the internal standard.

For the riboflavin measurement, 0.8 mL of culture broth was mixed well with 0.2 mL of 1 N NaOH. A 0.4-mL aliquot of the mixture was mixed with 1 mL of 0.1 M potassium phosphate buffer (pH 6.0); the optical density ($OD_{444\ nm}$) was then measured (11). The riboflavin concentration was calculated using an extinction coefficient of $1.04 \times 10^{-2}\ M^{-1}{\cdot}cm^{-1}$ (127 mg riboflavin/L/$OD_{444\ nm}$). In the case of the culture broth containing waste ABE, the sample was centrifuged at 500 × *g* for 15 min before measurement, and the supernatant was used for analysis.

The purity of waste ABE riboflavin was determined by HPLC (PU-980; JASCO Corp.) with a UV detector (UV-970; JASCO). The culture with 188 g/L waste ABE was used for investigating the distribution of riboflavin adsorbed in waste ABE. After 12 d of culture, 50 mL of culture broth containing waste ABE was centrifuged at 500 × *g* for 15 min. The supernatant was kept for analysis, and the resulting precipitated waste ABE was dried. Riboflavin extraction from dried waste ABE was carried out using a 1 M NaOH solution. The solution-extracted riboflavin and the supernatant of the culture broth were filtered through a 0.45-µm filter and injected into a Shim-pack column (VP-ODS; Shimadzu). Next, 5 mM sodium hexanesulfonate in 10 mM sodium phosphate (at pH 2.6) was mixed with 0.1 vol of acetonitrile, degassed, and used as the mobile phase for analysis at a flow rate of 1 mL/min. Authentic riboflavin (Sigma) was used as the standard.

During the cultivation, waste ABE was separated from the culture broth and dried at 105°C for 2 h and then photographed using a digital camera (FinePix 40i; Fuji Photo Film Co.). Mycelia, oil drops, waste ABE, and intracellular riboflavin crystals were observed using a microscope (IX-70; Olympus Optical Co.) equipped with a CCD camera (U-CMT; Olympus).

RESULTS AND DISCUSSION

Riboflavin production by A. gossypii *using waste ABE containing palm oil.* The palm oil contained in the waste ABE was used as the carbon source in the culture of *A. gossypii*. The initial concentrations of waste ABE were 62.5, 125, 188, and 250 g/L, which corresponded to 25, 50, 75, and 100 g/L palm oil, respectively. Figure 1 shows concentrations of residual oil and riboflavin, and riboflavin yield coefficient based on oil consumed. When 250 g/L waste ABE was used, 80% of the initial palm oil was consumed in 4 d, and 20% remained even after 6 more days (Fig. 1A). On the other hand, when 100 g/L pure palm oil was used as the reference, 61% of the initial palm oil was consumed and 39% remained even after 12 d of culture (Fig. 1a). In the case of waste ABE, oil droplets were not found in the culture broth, but in the case of pure palm oil, there was a large lump of palm oil, as the palm oil was partly solidified at 28°C due to its low m.p. (27–59°C)

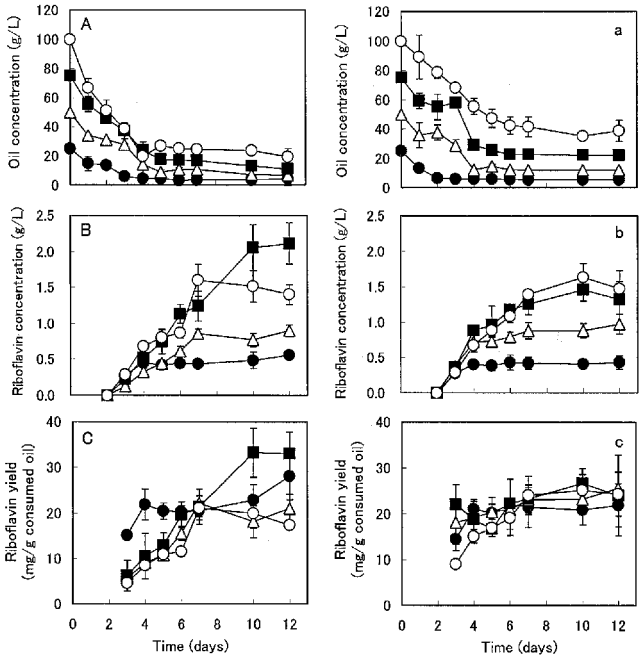


FIG. 1. Residual palm oil concentrations (A, a), riboflavin concentrations (B, b), and riboflavin yield coefficients (C, c). The amounts of waste activated bleaching earth (ABE) used in Figure 1A–C were (●) 62.5, (△) 125, (■) 188, and (○) 250 g/L. The amounts of pure palm oil used in Figure 1a–c were (●) 25, (△) 50, (■) 75, and (○) 100 g/L. Bars indicate SE for triplicate runs.

(data not shown). These results indicate that, in the culture without waste ABE, it is difficult for palm oil to be consumed by *A. gossypii* because the oil clumps. The initial oil-consumption rates for 250 g/L waste ABE and 100 g/L pure oil were 20.0 and 13.2 g/L/d, respectively.

When 188 g/L waste ABE was used, 2.11 g/L riboflavin was produced (Fig. 1B), which was the highest production rate among these experiments. The riboflavin concentrations increased with increases in the initial oil concentration, but at 250 g/L waste ABE, the maximum riboflavin concentration was only 1.60 g/L. In subsequent cultures with 250 g/L waste ABE, it was difficult to make the culture homogeneous due to the presence of pastelike materials that formed in the culture. However, when pure palm oil was used, the concentration of riboflavin produced after 10 d of culture increased with the increase in the initial oil concentration. The riboflavin concentration maximum was 1.64 g/L with an initial palm oil concentration of 100 g/L (Fig. 1b). The overall riboflavin production rates after 10 d of culture with waste ABE and pure palm oil were 0.21 and 0.16 g/L per day, respectively.

When 188 g/L waste ABE was used, the riboflavin yield coefficient based on weight of the consumed palm oil increased with culture time and reached 33 mg/g of palm oil consumed (Fig. 1C). The average yield coefficients in the culture using 62.5 and 125 g/L waste ABE were identically 22 mg of riboflavin/g of consumed oil. In the case of pure palm oil, the average riboflavin yield coefficient was 23 mg of riboflavin/g of consumed oil (Fig. 1c), which means that, when waste ABE was used, both the riboflavin production rate and riboflavin yield were about 45% higher than those when pure palm oil was used.

Behavior of waste ABE and A. gossypii *mycelia in culture.* Following inoculation of *A. gossypii* into the waste ABE medium (188 mg waste ABE/L) cultures were observed over time (Fig. 2). At a culture period of 1 d, mycelia grew well in the medium containing waste ABE (Fig. 2A) and the waste ABE containing palm oil was well suspended in the culture broth. After 3 d of growth, the mycelia took up FA from the palm oil, and mycelial filaments became thick due to the accumulation of FA (20) (Figs. 2B, b). With the progress of riboflavin biosynthesis, intracellular FA were converted to riboflavin, and riboflavin accumulated; sometimes the accumulated riboflavin formed intracellular crystals, as shown in Figure 2c, because of its low solubility in water (0.01 g/100 mL). When pure palm oil was used as the carbon source, intracellular crystals were observed inside the mycelia. In the culture with waste ABE, intracellular crystals were not observed, but the riboflavin produced was adsorbed directly onto the oil-depleted ABE, and the oil-depleted ABE became riboflavin-adsorbed ABE (Figs. 2C–E). Mycelia at a culture period of 5 d or longer contained small amounts of oil, and the cytosol did not contain riboflavin due to leakage (Figs. 2C, c, D, d). Finally, the mycelia were autolyzed, and the waste ABE used remained in the culture (Fig. 2E). However, when pure palm oil was used, spores were formed (Fig. 2e).

Change in appearance of waste ABE during cultivation. The color of waste ABE changed with culture time. ABE before use was a white powder (Fig. 3A), but after being used in the oil-refinery process, it became a dark brown, oily clay (Fig. 3B) due to the adsorption of crude oil, chlorophyll, and other colored materials contained in crude oil. The surface of waste ABE was completely smooth, as it was covered with palm oil (data not shown). However, with the progress of cultivation, *A. gossypii* cells consumed the palm oil contained in the waste ABE, resulting in a gradual fading of the black color with culture time, until finally waste ABE became oil-depleted ABE (Figs. 3C, D). Simultaneously, *A. gossypii* cells produced riboflavin; the oil-depleted ABE adsorbed riboflavin as it accumulated in the culture broth, resulting in a gradual change in the color of the oil-depleted ABE to yellow, finally becoming riboflavin-adsorbed ABE and an entirely yellow solid powder (Figs. 3E–G). When the riboflavin adsorbed was extracted, the yellow waste ABE changed to grayish claylike solid particles (data not shown).

Distribution of riboflavin and its purity. Riboflavin was partly adsorbed into the oil-depleted ABE; the rest remained in the culture broth or as intracellular crystals of mycelia. The intracellular crystals were not observed in the late culture because of mycelial autolysis (Figs. 2E, e). The riboflavin-adsorbed ABE was separated from the culture broth and the distribution of riboflavin adsorbed in the oil-depleted ABE was investigated, as shown in Figure 4. After 3 d of culture, 43% of the riboflavin produced in a 50-mL culture was adsorbed in the oil-depleted ABE, but after 12 d, 80% of the riboflavin had been adsorbed. The riboflavin content in the waste ABE increased gradually to 81.2 mg, which corresponds to 14 mg/g of oil-depleted ABE; whereas in the culture broth after the separation of the riboflavin-adsorbed

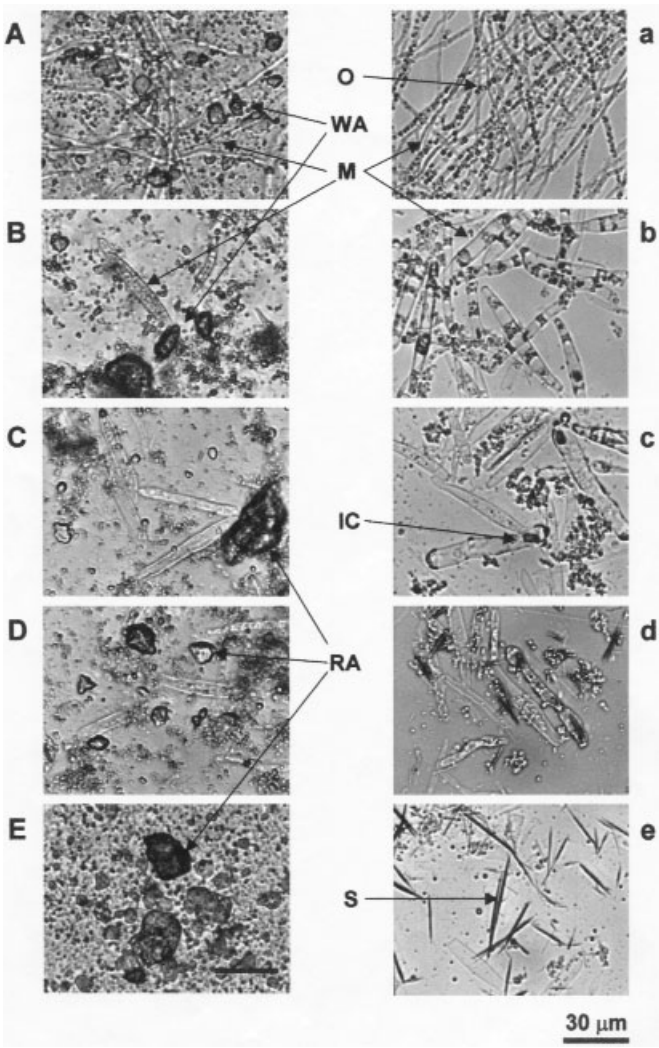


FIG. 2. Photomicrographs of the waste ABE and mycelia in the culture using waste ABE (A–E) and mycelia of *Ashbya gossypii* in the flask culture using pure palm oil (a–e). The waste ABE and mycelia were from the cultures with 188 g/L of waste ABE and 75 g/L of pure palm oil in Figure 1. Culture times were (A, a) 1; (B, b) 3; (C, c) 5; (D, d) 7; (E, e) 12 d. O, M, WA, IC, RA, and S indicate intracellular oil droplet, mycelia, waste ABE, intracellular riboflavin crystals, oil-depleted waste ABE absorbing riboflavin, and spores, respectively. For abbreviation see Figure 1.

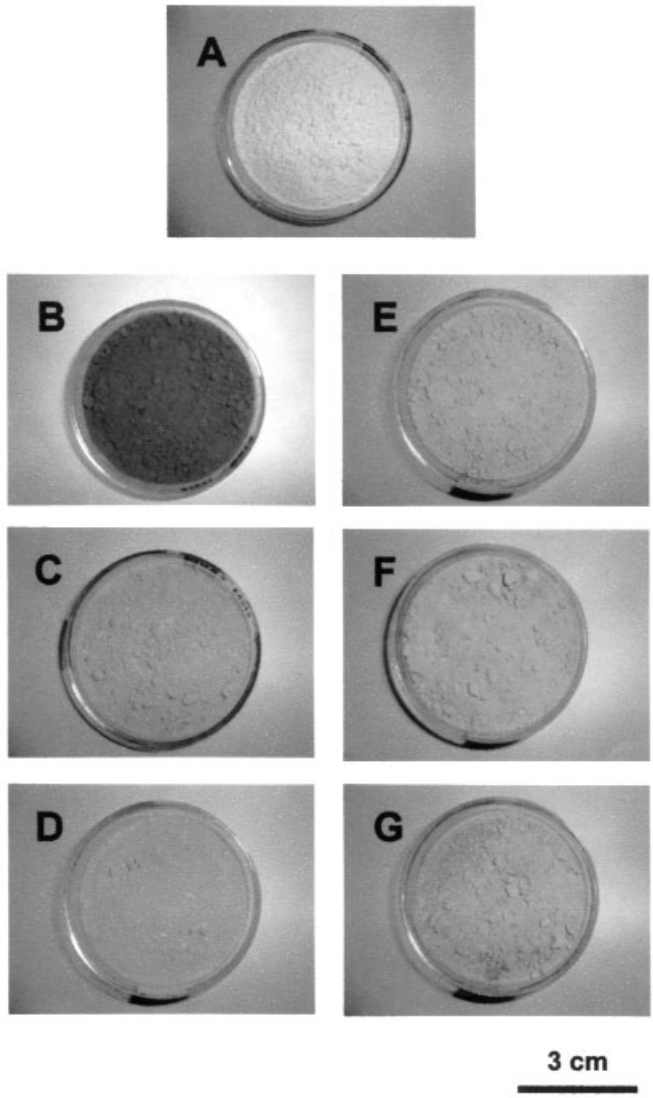


FIG. 3. Appearance of the waste ABE. After culture, the waste ABE was centrifuged and dried at 105°C for 2 h and then photographed with a digital camera. (A) Fresh ABE; (B) waste ABE. Culture times were (C) 1; (D) 3; (E) 5; (F) 7; (G) 12 d. For abbreviation see Figure 1.

ABE, the riboflavin content was only 20–23 mg, which might reflect a saturated level. These results indicate that 80% of the riboflavin produced in the culture is adsorbed into the oil-depleted ABE.

The riboflavin adsorbed in dried ABE after 12 d of culture was identified by HPLC, and then its purity was measured. The purity of the riboflavin eluted from riboflavin-adsorbed ABE (dry) was 64.5 ± 7.1% (n = 5), whereas that from the culture broth was 26.8 ± 1.0% (n = 5). This indicates that the riboflavin eluted from the riboflavin-adsorbed ABE was purer than that from the culture broth. The impure products were confirmed to originate from CSL, metabolites of *A. gossypii*, and waste ABE. From the results of HPLC analysis, 22% of the impurities were from CSL, 7.5% from waste ABE, and 70% might originate from the metabolites of *A. gossypii* but were not identified (data not shown).

There are two biotreatment methods for waste ABE: One is the bioconversion of its waste vegetable oil to biodiesel fuel (11), and the other is bioconversion to useful bioproducts using oil-degrading microorganisms. Various microorganisms have been used for riboflavin production, as shown in Table 1. Bacteria or yeasts use glucose as the carbon source and produce riboflavin at low yield (<1 g/L). *Eremothecium ashbyii* and *A. gossypii*, typical fungal riboflavin producers, use glucose and vegetable oil as carbon sources, respectively. The concentration of riboflavin produced by *E. ashbyii* is 1.0–2.0 g/L after 4 d of culture, but *A. gossypii* growing on pure vegetable oil yielded approximately 5 g/L, which is higher than that with other riboflavin producers. Ming *et al*. (18) reported that *A. gossypii* produced 1.1 g/L riboflavin after 6 d of culture using waste ABE containing rapeseed oil as the sole carbon source. In the case of waste ABE containing palm oil, saturated FA,

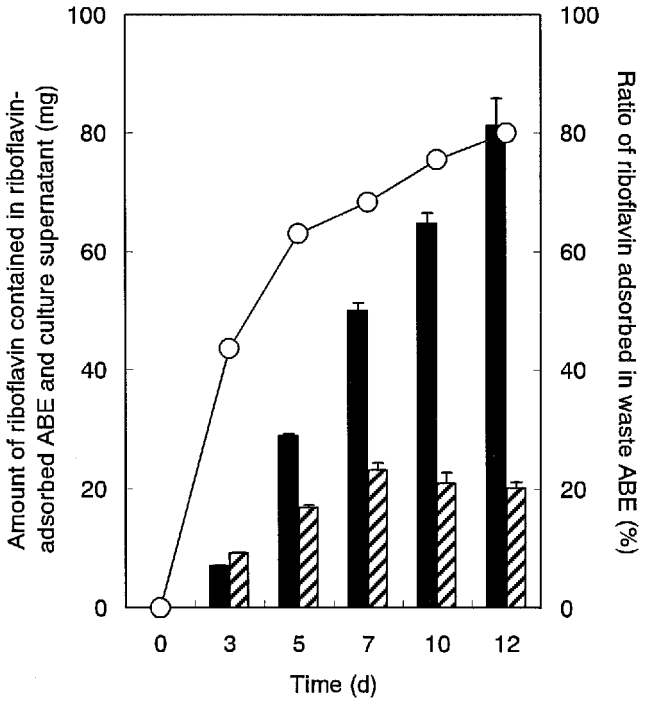


FIG. 4. Distribution of riboflavin in 50-mL culture scale. Solid and striped bars indicate riboflavin amounts contained in riboflavin-adsorbed ABE and in the culture supernatant, respectively. Fifty-milliliter cultures at 0, 3, 5, 7, 10, and 12 d were centrifuged at 500 × *g* for 15 min, and culture supernatant was separated from the wet riboflavin-adsorbed ABE. The latter was dried in a 105°C drying oven for 4 h, and a yellow powder was obtained. The riboflavin was extracted from the yellow powder using 10 vol of 1 N NaOH solution, and finally the riboflavin-containing solution was obtained. Concentrations of the culture supernatant and riboflavin-containing solution were measured, respectively. (○) The riboflavin percentage in the riboflavin-adsorbed ABE. Brackets indicate SE for triplicate runs.

constituting 60% of the total FA, are not considered essential for riboflavin production by *A. gossypii*. In this study, waste ABE containing palm oil discharged from oil refineries was found to be a good carbon source in riboflavin production by *A. gossypii*. The concentration of riboflavin produced in the culture with 188 g/L waste ABE was 2.1 g/L, which is 1.4-fold higher than that with 100 g/L pure palm oil (Fig. 1).

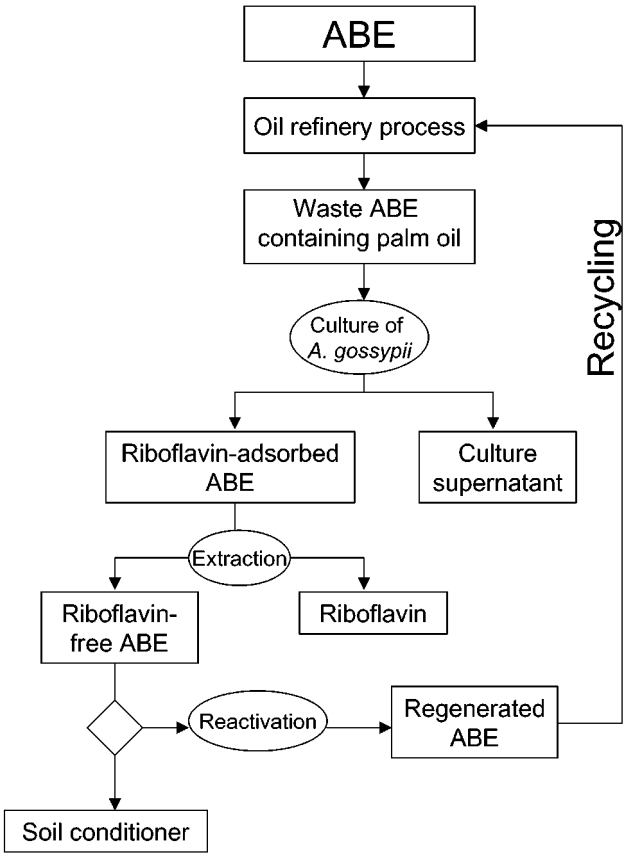
Eighty percent of the waste palm oil contained in waste ABE was consumed after 4 d of culture, and the oil-depleted ABE adsorbed most of the riboflavin produced by *A. gossypii* (Fig. 3). This changed the color of the ABE from black to yellow. The oil-depleted ABE absorbed 14 mg of riboflavin/g of oil-depleted ABE, which was four times as much as that remaining in the culture broth. Moreover, the riboflavin eluted from the oil-depleted ABE was purer than that from the culture broth. The amounts of impure compounds originating from the oil-depleted ABE were negligible in comparison with those from CSL and metabolites of *A. gossypii*. Based on the HPLC results, 22% of the impurities were likely from CSL and 70% might be from the metabolites (data not shown). We have previously shown that enhanced riboflavin production was achieved by adding a mineral support to a culture using soybean oil as a carbon source. A mineral support, a kind of ABE referred to as "mountain leather," which originated from sepiolite, has a stratified structure in which talc is regularly loaded on alternate layers that form a rectangular tunnel of 10.6 × 3.6 Å (19). When this support was added to the culture at a concentration of 1%, the riboflavin yield, based on the substrate consumed, was 1.6 times higher than that of the culture without the mineral support (20). This may be an example of extraction–fermentation that results in enhanced riboflavin production.

The implications of the current research are summarized in Scheme 1. The waste ABE containing palm oil was changed to oil-depleted ABE by *A. gossypii*. With the progress of cultivation, the riboflavin produced by *A. gossypii* was adsorbed onto the oil-depleted ABE and became riboflavin-adsorbed ABE. Eighty percent of the total amount of riboflavin in the culture was adsorbed onto the oil-depleted ABE and the remaining 20% stayed in the culture broth. Riboflavin adsorbed in ABE could easily be purified for use in the medicine, food, and fodder industries. The purity of the desorbed riboflavin was 65%,

TABLE 1
Summary of Riboflavin Productivity[a]

Strain	Carbon sources	Nitrogen sources	Maximum riboflavin concetration (g/L)	Culture period	Reference
Bacterium					
Bacillus subtilis	Glucose	—	0.08	0.3 h	12
Yeasts					
Candida flareri	Glucose, fructose	Urea, glycine, serine, threonine	0.6	—	13
Candida guilliermondii	Liquid brewery waste	—	0.2	3 d	14
Mycobacterium phlei	Beet molasses	Peptone	0.1	6 d	15
Fungi					
Eremothecium ashbyii	Glucose, molasses	CSL, yeast extract, meat extract	1.0–2.0	4 d	16
Ashbya gossypii	Corn oil, soybean oil	CSL, yeast extract, collagenous proteins	5.0	—	17
	Soybean oil, bone fat	CSL, gelatin	5.0	8 d	9
	Waste ABE containing rapeseed oil	CSL, gelatin	1.1	6 d	18
	Waste ABE containing palm oil	CSL, gelatin	2.1	10 d	This work

[a]CSL, corn steep liquor; ABE, activated bleaching earth.



SCHEME 1

and that of riboflavin in the culture broth was only 27%. After de-sorption of riboflavin, the riboflavin-free ABE may be used as a soil conditioner. Otherwise, by treating with sulfuric acid, the riboflavin-free ABE may be used as an adsorbent in the oil-refinery process, although the reactivation process is costly. This research carried out using waste ABE provides a model for the bioconversion of waste resources to a value-added bioproduct and/or recycling of waste resources. To our knowledge, this is the first report on riboflavin production by *A. gossypii* with waste ABE containing palm oil. Further research is required for the scaling up of reactors and for establishing an economic process for the reutilization of waste ABE.

ACKNOWLEDGMENTS

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